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Serum lathosterol concentration is an indicator of whole-body cholesterol synthesis in humans[1]

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Abstract The power of serum lathosterol concentration as an indicator of whole-body cholesterol synthesis was investigated in 47 human volunteers consuming two diets differing in fatty acid composition. The cholesterol balance (fecal excretion of neutral and acid steroids minus cholesterol intake) was strongly correlated with the serum level of total (free plus esterified) lathosterol and also with the ratio of serum lathosterol over serum cholesterol, both on a diet rich in polyunsaturated fatty acids (r = 0.74 for the ratio) and one containing mainly saturated fatty acids (r = 0.70 for the ratio). In a subgroup for which the serum levels of free lanosterol and other free methylsterols were also quantitated, the correlations of these levels (expressed relative to serum free cholesterol) with the cholesterol balance were lower than that found for total lathosterol (expressed relative to serum total cholesterol). A further corroboration was obtained by measuring the lathosterol/cholesterol ratio in 20 patients with familial hypercholesterolemia before and during treatment with the hydroxymethylglutaryl coenzyme A reductase inhibitor Mk-733. The ratio was lowered by 47% during drug treatment, suggesting a significant decrease of the cholesterol balance in these patients. We conclude, from the various indicators proposed to monitor whole-body cholesterol synthesis, that the lathosterol/cholesterol ratio in serum appears preferable with respect to indicative power and ease of quantitation. — **Kempen, H. J. M., J. F. C. Glatz, J. A. Gevers Leuven, H. A. van der Voort, and M. B. Katan.** Serum lathosterol concentration is an indicator of whole-body cholesterol synthesis in humans. *J. Lipid Res.* 1988. **29:** 1149–1155.

Supplementary key words cholesterol precursors • cholesterol balance • cholesterol synthesis inhibition (man) • Mk-733, simvastatin

The classical method of assessing the rate of whole-body cholesterol synthesis in humans, without resorting to the use of radioisotopes, comprises measurement of the cholesterol balance, i.e., fecal excretion of cholesterol plus its acidic (1) and neutral (2) metabolites, minus cholesterol intake. Although this method is well established, it has the disadvantage of being laborious and is not suited to detecting changes in cholesterol synthesis occurring within the time-scale of less than a few days. Moreover, the method requires that the subjects be in a steady state with regard to cholesterol metabolism. For these reasons, alternative methods have been sought to monitor whole-body cholesterol synthesis. Serum levels of various precursors along the cholesterol synthesis pathway have been proposed for that purpose, namely (in historical order) free methylsterols (3–5), squalene (3, 6, 7), and mevalonic acid (8, 9). Levels of these precursors were shown to correlate significantly with the cholesterol balance in conditions with greatly varying rates of cholesterol synthesis and to fluctuate with a diurnal rhythm (4, 5, 9, 10). Plasma squalene, however, has now been established to reflect the plasma VLDL concentration rather than the rate of whole-body cholesterol synthesis (11, 12).

Miettinen (13) and Vuoristo and Miettinen (14) reported recently that the serum level of 7-lathosterol (5α-cholest-7-ene-3β-ol) in humans decreased upon expansion and increased upon depletion of the bile acids pool, in a more pronounced way than free methylsterols. We have investigated whether the serum level of lathosterol was correlated with the cholesterol balance in healthy volunteers on two controlled diets. The study was originally designed to assess the effect of a change in dietary fatty acid composition on cholesterol synthesis. The specific effects induced by the diet change will be reported extensively elsewhere. Here we wish to report that the ratio of serum lathosterol over serum cholesterol strongly correlates with the cholesterol balance under both dietary conditions. Moreover, this ratio fell in patients with familial hypercholesterolemia during treatment with a potent HMG-CoA reductase inhibitor.

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; ACAT, acylcoenzyme A:cholesterol acyltransferase; LCAT, lecithin:cholesterol acyltransferase.

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MATERIALS AND METHODS

Subjects and treatments

The first part of the study was carried out with 47 healthy volunteers living in or around Wageningen, who participated after being informed about the purpose and design of the study. They formed a composite of one group of 23 and another of 24 subjects. Group 1 consisted of 14 men and 9 women, most of them students and university staff. All had participated in three previous controlled dietary trials (15, 16). Their characteristics at entry were (means ± SD): age 34 ± 13 yr, height 178 ± 9 cm, weight 71.4 ± 9.5 kg, and body mass index 22.5 ± 2.2 kg/m^2. Group 2 consisted of 10 men and 14 women with a high habitual consumption of eggs. All of them had participated earlier in two outpatient trials on the effect of dietary cholesterol on serum lipids (17, 18), and six had participated in a third controlled trial (19). Their characteristics at entry into the trial were: age 54 ± 13 yr, height 172 ± 7 cm, weight 73.7 ± 15.2 and body mass index 24.4 ± 2.8 kg/m^2.

The baseline levels of serum total cholesterol, HDL cholesterol, and triglycerides for these 47 subjects were 5.66 ± 1.21, 1.42 ± 0.32, and 1.32 ± 0.75 mmol/L, respectively, determined in fasting blood samples taken 2–4 weeks before starting the experiment.

The subjects in Group 1 first received a mixed natural diet that was high in polyunsaturated and low in saturated fatty acids for 3 weeks (high P/S diet), and then changed to a diet rich in saturated and poor in polyunsaturated fatty acids for another 3 weeks (low P/S diet). For the subjects in Group 2 the order of these diets was reversed. The intake of total fat and of cholesterol did not change from one dietary period to another. The diets were prepared and supplied at the Department of Human Nutrition as described (20). Nutrient and sterol compositions of the diets were assessed by analysis of duplicate portions for one imaginary person of average energy intake on each diet. These compositions are given in **Table 1.** The food was found to contain a number of known and unidentified sterols, but lathosterol was not detected. Mean body weight (± SD) at the end of the high P/S diet was 0.08 ± 0.79 kg higher than at the end of the low P/S period.

Blood samples were taken via the antecubital vein twice during the third week of each dietary period from fasting subjects. Portions of serum from these two samples were mixed and used for determination of serum cholesterol, lathosterol, and free methylsterol levels. Feces were collected during the last 5 days of each period. Radio-opaque markers were taken orally by the subjects (20/day from 10 days before until the end of each fecal collection period), and the recovery of these was used to correct for variations in fecal flow, as described (20). The stools were frozen as soon as possible after being passed, and stored at −20°C. At the end of the experiment, the fecal collections from each person were combined per diet period, homogenized, freeze-dried, and again stored at −20°C.

TABLE 1. Mean daily nutrient intake of the 47 subjects during the two dietary periods, according to duplicate portion analysis[a]

Nutrient		High P/S diet	Low P/S diet
Total energy[b]	(MJ)	11.6	11.5
Protein	(en %)	13.2	14.2
Fat	(en %)	45.0	44.5
Saturated	(en %)	10.6	23.4
Monounsaturated	(en %)	11.7	14.3
Polyunsaturated[c]	(en %)	20.8	5.2
Carbohydrates	(en %)	39.9	39.4
Alcohol	(en %)	2.0	2.0
Cholesterol	(mg/day)	483	456
Campesterol	(mg/day)	65	35
Stigmasterol	(mg/day)	47	22
Sitosterol	(mg/day)	369	153
Unidentified sterols	(mg/day)	277	103
Lathosterol		not detectable	

[a]Includes calculated contribution of free-choice items to macronutrient and cholesterol intake (16).
[b]One MJ equals 239 Kcal.
[c]Mainly linoleic acid.

The second part of the study consisted in determining lathosterol and cholesterol concentrations in the fasting serum of 20 patients heterozygous for familial hypercholesterolemia (FH) before and 1 and 6 months after starting treatment with the HMG-CoA reductase inhibitor Mk-733. These patients took part in an earlier clinical trial with this drug (21). In the present study they received 20 mg of the drug per day during the first two days and 40 mg per day during the next 5 months. Characteristics of these patients and effects of these treatment schedule on their serum (apo)lipoprotein levels are reported elsewhere (22).

Analytical techniques

Cholesterol and plant sterol contents of the consumed food (23) and the neutral steroid and bile contents of the feces (24) were determined by capillary gas–liquid chromatography. Feces from each subject were analyzed in duplicate. In separate tests the coefficient of variation was about 2.5% for dietary cholesterol and less than 5% for the total amount of excreted neutral steroids and bile acids. The rate of whole-body cholesterol synthesis was calculated as the sum of the excreted endogenous steroids and bile acids minus the cholesterol intake (15, 16).

Total (free plus esterified) serum cholesterol was determined colorimetrically by the method of Abell et al. (25). Free cholesterol was assayed with a commercially available test kit (Boehringer Mannheim, cat. no. 310328).

For the assay of total lathosterol, duplicate samples of 200 μl serum were mixed each with 1 ml 0.3 M NaOH in

90% ethanol, and incubated for 60 min at 37°C. After addition of 1 ml distilled water, the nonsaponifiable lipids were extracted with 4 ml hexane. Campesterol (6.75 μg; Applied Sciences) was added as internal standard to each of the serum samples.[4] The nonsaponifiable lipids were converted into their trimethylsilyl ethers using Sil-Prep® (Alltech Associates, Eke, Belgium). Lathosterol was quantitated by gas–liquid chromatography, using a Packard 433 chromatograph equipped with a 25 m × 0.22 mm (i.d.) fused silica capillary column with CP-Sil-5CB as the stationary phase (Chrompack, Middelburg, The Netherlands). Hydrogen was used as the carrier gas; the column was kept at a constant temperature of 235°C. Detection was by flame ionization. Response factors were assumed to be equal among the various steroids. Pure lathosterol (Steraloids, Wilton, NH) had a relative retention time of 1.12 with respect to cholesterol and of 0.85 with respect to campesterol. The procedure was found to have an intraassay coefficient of variation of 2% (n = 5).

Free lanosterol and the sum of free methylsterols in serum were determined by a procedure modified from that described by Miettinen (4, 26). Analyses were carried out for a subset of 19 subjects from whom enough serum was available. Epicoprostanol (3 μg) was added as internal standard to 1 ml serum, and total lipids were extracted by the method of Folch, Lees, and Sloane Stanley (27). The extract was applied on a 20 × 20 cm thin-layer silica gel 60 plate (Merck, West Germany, art. 5748), one sample per plate. To separate the various lipid classes, the plate was first developed in hexane–toluene 90:10 (by vol), and then in heptane–diethyl ether 55:45 (by vol). Lipids were localized by spraying with Rhodamine. The free methylsterol band was scraped between the positions of cholesterol and α-tocopherol reference spots. The free methylsterols were eluted from the silica with diethyl ether, and the solvent was evaporated. Quantitation by gas–liquid chromatography was done as described above for lathosterol, including prior derivatization into trimethylsilyl ethers. Free lanosterol (denoted as peak IV by Miettinen (4, 5)) was identified as such in serum extracts by mass spectrometry, and had a relative retention time of 1.81 with respect to epicoprostanol. Other free methylsterols, denoted by Miettinen as peaks I, III, II, and V, respectively, had retention times of 1.52, 1.66, 1.77, and 1.92 with respect to epicoprostanol. Repeated determinations on a pooled sample of human serum revealed intraassay coefficients of variation of 9% (n = 6) for free lanosterol.

Statistical methods

Pearson correlation coefficients and linear regression parameters (slope, intercept, and standard error of the estimate, denoted as a, b, and s, respectively, in the formula below) were calculated, and statistical significance of differences between these parameters for the two diet periods was evaluated by conventional methods (28). The 95% confidence intervals of the correlation coefficients were obtained using Fisher's Z-transformation. The 95% confidence intervals of the cholesterol balance (y) predicted from the lathosterol/cholesterol ratio (x) were found using the formula

$$y = a \cdot x + b \pm t_{(0.05;\, n-2)} \cdot s \cdot \sqrt{1/n + \frac{(x - \bar{x})^2}{\sum_i (x_i - \bar{x})^2}} \quad Eq.1$$

in which $t_{(0.05;\, n-2)}$ is the tabulated value at $P = 0.05$ (two-tailed) and n − 2 degrees of freedom (i.e., 2.15), and $\bar{x}$, n and $\sum_i (x_i - \bar{x})^2$ are obtained from the data of the diet study.

RESULTS

Relation between cholesterol balance and serum lathosterol level

This study was designed originally to document the effect of a change in dietary fatty acid composition on the rate of cholesterol synthesis. However, it is the purpose of the present study to focus on the serum levels of cholesterol precursors as indicators of cholesterol synthesis; the responses of serum sterols and cholesterol balance toward the change in dietary fatty acid composition will be described and discussed extensively elsewhere (Glatz, J. F. C., and M. B. Katan, manuscript in preparation). Nevertheless, it was deemed desirable to provide these levels in summary form, to be read only as background information.

Table 2 lists the mean serum concentrations of cholesterol and lathosterol, the ratio of lathosterol over cholesterol, as well as the whole-body cholesterol balance, for the 47 participants in the third week of the two diet periods. For a subgroup of 19 persons, the serum levels of free cholesterol, free lanosterol, and sum of free methylsterols are also given. The mean values for serum sterols and for the cholesterol balance are in good agreement with those reported previously (1–5, 15, 16, 20) for adult humans on average Western diets. It is to be noted that the level of lathosterol is an order of magnitude higher than that of the free methylsterols. It is shown, furthermore, that the levels of the various cholesterol precursors and the rate of whole-body cholesterol synthesis vary to a greater extent than the serum levels of cholesterol.

The concentration of total lathosterol in serum correlated very strongly with the cholesterol balance, both on

[4] The amount of campesterol present in 200 μl of five sera from each diet group was less than 1% of the amount of added campesterol. However, the use of another steroid not occurring in serum, such as 5α-cholestane, as internal standard is to be preferred.

TABLE 2. Mean levels of serum sterols and their ratios and the cholesterol balance[a]

Total cholesterol (mmol/L)	5.53 ± 1.04
Free cholesterol (mmol/L)	1.51 ± 0.32
Total lathosterol (μmol/L)	5.32 ± 2.05
Lathosterol/cholesterol (μmol/mmol)	0.96 ± 0.34
Free lanosterol (μmol/L)	0.30 ± 0.11
Sum of free methylsterols[b] (μmol/L)	1.38 ± 0.54
Free lanosterol/free cholesterol (μmol/mmol)	0.20 ± 0.06
Sum of free methyl sterols/free cholesterol (μmol/mmol)	0.91 ± 0.34
Cholesterol balance (mmol/day)	1.69 ± 0.78

[a]Measured in the last week of a 3-week period of controlled feeding of a diet high (high P/S) or low (low P/S) in polyunsaturated fatty acids. Means ± SD of 47 or, for serum free sterols, of 19 subjects.
[b]Assumed to have a molecular weight of 400.

the high P/S ($r = 0.76$; $P < 0.001$) and on the low P/S ($r = 0.73$; $P < 0.001$) diet. However, a significant positive correlation was also observed between serum lathosterol and serum cholesterol for both diet periods ($r = 0.34$ for the high P/S and 0.29 for the low P/S diet, both $P < 0.05$). Therefore, lathosterol was adjusted for differences in total cholesterol by dividing the lathosterol concentration by the cholesterol concentration. As shown in **Fig. 1,** the lathosterol/cholesterol ratio was still highly correlated with the cholesterol balance, with $r = 0.74$ for the high and 0.70 for the low P/S diet period.

It has been reported before (29) that the rate of cholesterol synthesis correlates with body weight. This was confirmed in the present study: correlation coefficients of 0.56 ($P < 0.001$) existed between body weight and mean cholesterol balance at the end of the two diet periods. Nevertheless, even when the cholesterol balance was expressed per kg body weight, it correlated significantly with the lathosterol/cholesterol ratio ($r = 0.65$ and $r = 0.58$, both $P < 0.001$, for the high P/S and low P/S period, respectively).

It might seem from inspection of Fig. 1A and 1B that the relation between the lathosterol/cholesterol ratio and the cholesterol balance was dissimilar in the two dietary situations. This was tested by comparing the slopes and intercepts of the regression lines obtained for the two diet periods. A t-value of 1.18 ($P = 0.25$) was calculated for the difference between slopes, and of -0.49 ($P = 0.63$) for the difference between intercepts (on the assumption of equal slopes; see ref. 28), which gives no support to the hypothesis that the regression lines are different.

For the subgroup of 19 persons for whom the levels of lanosterol and free methylsterols were also quantitated, the lathosterol/cholesterol ratio again correlated significantly with the cholesterol balance, but the free lanosterol/free cholesterol ratio and the free methylsterols/free cholesterol ratio did not. The correlation coefficient for the lathosterol/cholesterol ratio was significantly different from that for the free lanosterol/free cholesterol ratio (**Table 3**).

Effect of Mk-733 on serum sterol levels

In 20 patients heterozygous for familial hypercholesterolemia, treatment for 1 month with the cholesterol synthesis inhibitor Mk-733 lowered the level of cholesterol on average by 30% and that of lathosterol by 64%, so that the lathosterol/cholesterol ratio fell by 47% (**Table 4**). These effects did not materially change during the next 5 months of treatment. In a subset of 10 patients for whom serum free methylsterols were obtained as well, the lathosterol level tended to decrease more (by 54 ± 10%) than the level of free lanosterol (which fell by 42 ± 23%) after 1 month treatment, although the difference between these effects was not statistically significant.

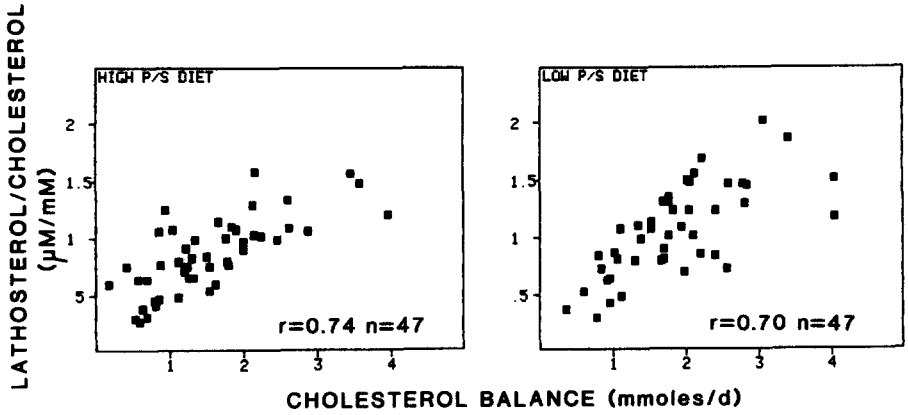

Fig. 1. Relationship between the lathosterol/cholesterol ratio in serum and the cholesterol balance in 47 subjects on a diet high (left-hand panel) or low (right-hand panel) in polyunsaturated fatty acids. The regression lines for cholesterol balance (Y) on lathosterol/cholesterol ratio (X) are: Y = 1.90 X − 0.08 (standard error of estimate 0.58) for the high P/S period, and Y = 1.50 X + 0.24 (standard error of estimate 0.60) for the low P/S period.

TABLE 3. Pearson correlation coefficients (r), and 95% confidence intervals (CI)[a]

	r	95% CI
Total lathosterol/total cholesterol	0.663	0.435–0.811
Free lanosterol/free cholesterol	0.052	−0.282–0.374
Sum of free methylsterols/free cholesterol	0.212	−0.148–0.522

[a]For the associations of cholesterol balance with the ratio total lathosterol/total cholesterol, the ratio free lanosterol/free cholesterol, or the ratio (sum of free methylsterols)/free cholesterol in 19 subjects on diets high or low in polyunsaturated fatty acids (data from both diet periods were used, yielding 38 pairs in each calculation).

The 95% confidence interval for the rate of whole-body cholesterol synthesis was estimated in these patients from their mean lathosterol/cholesterol ratio. For that purpose, the slope, intercept, and standard error of the estimate, as found in the volunteer study, were substituted in equation 1. This resulted in a prediction equation having the form:

$$y = 1.66 \cdot x + 0.08 \pm 1.27\sqrt{1/47 + \frac{(x - 0.96)^2}{6}}$$

Accordingly, the predicted intervals for the whole-body cholesterol synthesis were 1.32–1.70 mmol/day before, and 0.59–0.97 mmol/day at 6 months after starting the Mk-733 medication. This indicates a statistically significant decrease in whole-body cholesterol synthesis.

DISCUSSION

The rationale for using the serum level of cholesterol precursors as an indicator for cholesterol synthesis lies in the assumption that these compounds in their unesterified form "leak" into plasma lipoproteins at a rate proportional to that of their formation in the cholesterol synthetic pathway. Only the unesterified methylsterols in serum are considered to reflect cholesterol synthesis, since their esterification can only take place in the liver by ACAT (30) and not by LCAT in the plasma (31). Indeed, only the free and not the esterified methylsterols were found to vary with a diurnal rhythm (4). The amount of esterified methylsterols (which is less than 20% of the total (4)) has been proposed as an indicator of hepatic ACAT activity or VLDL secretion (32). In contrast, lathosterol was present for 50% or more in the esterified form (14, confirmed in our own laboratory) and probably is esterified by LCAT in the serum. For this reason, total rather than free lathosterol in serum may be expected to reflect "leakage" from cellular sterologenesis.

Furthermore, Miettinen (3–5) has advocated expression of the serum levels of precursors relative to the level of free or total cholesterol as a way to correct for differences in these levels that would occur as a mere consequence of a different number of acceptor (lipoprotein) particles in the serum compartment. We have followed this practice in this report, supported by the finding of a significant, although low, correlation between serum lathosterol and cholesterol levels. Division of the lathosterol values by the cholesterol concentrations did not, however, materially affect the high correlation coefficients with the cholesterol balance.

The potential use of the serum level of lathosterol as indicator of the whole-body cholesterol synthesis was first discovered in a study with a relatively small number of patients, in which the actual rate of cholesterol synthesis was not determined (13). The present study, done with a large group of healthy volunteers on two different diets, shows directly that lathosterol and the lathosterol/cholesterol ratio are indeed good monitors of whole-body cholesterol synthesis within the range of synthetic rates occurring in healthy people on normal Western diets, in a manner that is apparently independent of the fatty acid composition of the diet. To our knowledge, this capacity as an indicator has not been demonstrated for either methylsterols or mevalonate.

Miettinen et al. (13, 14) observed that lathosterol changed in a more pronounced manner than free methylsterols upon manipulating the enterohepatic circulation. We add the following evidence that serum lathosterol is superior to serum free methylsterols as an indicator of cholesterol synthesis. First, in 19 participants of the diet study, the cholesterol balance correlated significantly better with the lathosterol/cholesterol ratio than with the free lanosterol/free cholesterol ratio (Table 3). Secondly, in a previous study of 41 subjects on diets high or low in dietary cholesterol (given as egg yolks) (15, 16) we found a low correlation ($r = 0.30$, $P < 0.05$) between the cho-

TABLE 4. Serum levels (means ± SD) of lathosterol and cholesterol in 20 patients with familial hypercholesterolemia before and during treatment with Mk-733

	Before		One Month after Start		Six Months after Start
Lathosterol (μmol/L)	9.6 ± 4.0	*[a]	3.5 ± 1.4		2.9 ± 1.8
Cholesterol (mmol/L)	11.3 ± 1.9	*	7.9 ± 1.3	*	7.0 ± 1.4
Latho/chol (μmol/mmol)	0.86 ± 0.36	*	0.46 ± 0.19		0.42 ± 0.26

[a]Significant difference between values on either side of the asterisk.

lesterol balance and the serum level of free lanosterol during the low, but none at all during the high cholesterol period (Katan, M. B., A. C. Beynen, and C. M. van Gent, unpublished results). The lack of correlation in the latter period might be related to the fact that egg yolks contain considerable amounts of lanosterol (350 ± 30 μg per egg yolk (n = 4); Kempen, H. J. M., and H. A. van der Voort, unpublished observations), of which a fraction possibly was absorbed. This factor may be involved in the present study as well. In contrast, lathosterol was not detected in the food in the present study.

Serum lathosterol or the lathosterol/cholesterol ratio are not only superior as indicators, but quantitation of total lathosterol is also considerably simpler than that of free methylsterols, due mainly to the fact that the concentration of lathosterol is an order of magnitude higher and a laborious sample workup using thin-layer chromatography is not required. Plasma mevalonate was recently proposed as a suitable indicator of cholesterolgenesis (8, 9), but for its assay one has to resort to a relatively tedious radio-enzymatic procedure.

The lathosterol/cholesterol ratio was also measured in 20 patients heterozygous for familial hypercholesterolemia. The mean value in these patients was not significantly different from that in the 47 volunteers in the diet study. This is in line with previous studies (33, 34) in which adult FH heterozygotes were found to have normal rates of cholesterol synthesis, as measured by more direct methods. Furthermore, upon treatment of these patients with the HMG-CoA reductase inhibitor Mk-733, the lathosterol/cholesterol ratio dropped by about 50%. When the regression parameters found in the volunteer study were used to estimate the 95% confidence interval for the cholesterol balance in the patients before and on the drug, the drug caused a significant drop in the predicted value. This would be in contrast with a study by Grundy and Bilheimer (35) in which mevinolin did not lead to a significant decrease in cholesterol balance in five FH heterozygotes.

Addendum. After completion of this manuscript, Björkhem et al. (36) reported that serum levels of free methylsterols and of free and total lathosterol were all highly correlated with the hepatic HMG-CoA reductase activity in human patients subjected to gall bladder surgery. A reason why the values for the correlation coefficients were higher than found in our study may be that these workers included not only untreated patients but also patients pretreated with cholestyramine or with chenodeoxycholic acid. This probably results in a much wider range of cholesterol synthetic rates than was the case in our group of volunteers.

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